The Glucagon-like peptide-1 receptor agonist, exendin-4, ameliorated gastrointestinal dysfunction in the Wistar Kyoto rat model of Irritable Bowel Syndrome.

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Running title: GLP-1 mollifies IBS-like symptoms.

Number of words (excluding abstract, key points, references, acknowledgements and legends): 4,802

For submission to: *Neurogastroenterology and Motility*.

Abstract:

Background: Glucagon-like peptide-1 (GLP-1) is beneficial in relieving pain-related symptoms of Irritable bowel syndrome (IBS), a prevalent, multifactorial functional bowel disorder characterized by diarrhea and/or constipation, abdominal bloating and pain. Activation of myenteric neurons have been implicated in the inhibitory effects of GLP-1 on gastrointestinal motility, however the mechanisms of action underlying this are not clear.

Methods: A rat model of IBS was used to examine physiological changes evoked by intraperitoneal administration of a GLP-1 receptor agonist, exendin-4. Behavioral and physiological analysis of stress-sensitive Wister Kyoto (WKY) rats was used to determine if administration of exendin-4, in the presence, or absence of neutralizing interleukin-6 receptor monoclonal antibodies, modified IBS-like symptoms. Immunofluorescence, calcium imaging and Western blotting techniques were used to investigate the potential role of enteric neural plexi and tight-junction protein expression in this effect.

Key results: Consistent with the expression of GLP-1 and interleukin-6 receptors in both submucosal and myenteric ganglia, exendin-4 and interleukin-6 stimulated calcium responses in these neurons. *In vivo* administration of exendin-4 normalized stress-induced defecation and visceral pain sensitivity in WKY rats. No additional changes were noted in rats co-treated with exendin-4 and anti-interleukin-6 receptor antibodies. Mucosal expression of occludin, a tight junction protein was decreased by exendin-4. Centrally-regulated anxiety-like behaviors were not modified.

Conclusions & inferences: These data suggest that intraperitoneal injection of exendin-4 improves bowel dysfunction in WKY rats without impacting on centrally-

regulated anxiety-like behaviors. Modulation of enteric neuronal function and tight junction expression appear to underlie the functional benefits of this intervention.

Keywords: Exendin-4, glucagon-like peptide-1, Wister Kyoto, colorectal distension, open-field, interleukin-6, stress.

Key points

- Glucagon-like peptide-1 (GLP-1) is beneficial in alleviating symptoms in patients with Irritable Bowel Syndrome (IBS), but the mechanisms underlying this effect remain unclear.
- In Wister Kyoto (WKY) rats, which mimic symptoms of IBS, intraperitoneal injection of a GLP-1 receptor agonist, alleviated stress-induced defecation and visceral pain sensitivity, but did not impact on centrally-regulated anxiety-like behaviors.
- Activation of peripheral GLP-1 receptors are likely to underlie the amelioration of IBS-like symptoms by GLP-1.

The prevalent functional bowel disorder, Irritable Bowel Syndrome (IBS), is characterized by chronic episodic bouts of abdominal pain, bloating and altered bowel habit, which may manifest as constipation, diarrhea or both [1]. Although our understanding of the underlying pathophysiology has improved in recent times [2], a lack of sensitive and specific biomarkers continues to hamper diagnosis and treatment. Factors such as food, bile acids, antibiotics and infections, gender and adverse psychosocial events, particularly in early childhood, are all implicated in alterations in the gut epithelial barrier [3]. This may result in increased intestinal leakiness causing immune activation and neuroendocrine responses. Combined with microbial changes this results in abnormal gut secretory and sensorimotor function [4-6].

Post-prandial exacerbation of symptoms is problematic in many IBS patients [7-9], thus satiety-related hormones may have a role in IBS pathophysiology [10]. Glucagon-like peptide-1 (GLP-1), which is released from intestinal L-cells in response to the arrival of nutrients in the intestinal lumen [11], is a circulating satiety hormone with a peak concentration that coincides with IBS post-prandial symptom manifestation [12]. In addition to being an incretin hormone, GLP-1 also modifies gut function, inhibiting small intestinal secretion [13], gastrointestinal (GI) motility, gastric emptying and the migrating motor complex in both healthy controls and in patients with IBS [14-16]. Furthermore, administration of a GLP-1 mimetic resulted in relief from gut spasms and pain in IBS patients [17,18]. In a rat model of IBS, colonic mRNA and protein expression of GLP-1Rs was suppressed in diarrhea predominant-IBS and elevated in constipation-predominant IBS [19], suggesting it could underlie divergence of symptoms in IBS patients.

Prior inflammatory gastroenteritis confers an increased susceptibility to develop IBS [20] and IBS mucosal biopsies exhibit increased numbers of T-cells, intra-epithelial lymphocytes and mast cells [21]. We have previously demonstrated that colonic secretions from the stress-sensitive Wistar Kyoto (WKY) rat model of IBS stimulates naïve submucosal neurons to a greater extent than secretions from low anxiety Sprague Dawley (SD) controls; an effect mediated in part by the pro-inflammatory cytokine, interleukin (IL)-6 [22]. Circulating levels of inflammatory cytokines such as IL-6 have reproducibly been found to be raised in IBS patients [23-25], particularly in diarrhea-predominant IBS [26]. However, more than just being a biomarker of IBS, IL-6 has functional effects in the GI tract, activating submucosal secretomotor neurons [27,28] and modulating mucosal ion transport and epithelial permeability [29,30]. Moreover, neutralization of peripheral IL-6R signaling ameliorated visceral hypersensitivity and stress-induced defecation in the WKY rat model of IBS [31]. As cross-talk between immune, endocrine and neural systems is likely to underlie the multifactorial nature of IBS [32], this study investigated if exendin-4 (Ex-4), a GLP-1 receptor (GLP-R) agonist, improves bowel dysfunction in WKY rats and determined if co-treatment with neutralizing monoclonal IL-6 receptor (IL-6R) antibodies modified the outcome.

Materials and Methods

Ethical approval

All experiments were in full accordance with the European Community Council Directive (86/609/EEC) and the local University College Cork animal ethical committee. Rats were sacrificed by CO_2 overdose and decapitation (#2011/015).

Animals and tissue collection.

To avoid interference from the female hormonal cycle in the assessment of the GI hormone, GLP-1, in bowel dysfunction, male Sprague Dawley (SD) control and stress-sensitive Wister Kyoto (WKY) rats (8-12 weeks old, 200-250g), purchased from Envigo, Derbyshire, UK, were used. Animals were group-housed 5 per cage and maintained on a 12/12 hour light-dark cycle (08.00-20.00) with a room temperature of 22±1^{o}C. Food and water were available *ad libitum*. WKY rats are a stress-sensitive strain, which exhibit a high anxiety phenotype, visceral hypersensitivity and increased stress-related defecation [33-35]. This model of brain-gut axis dysfunction has been validated as a rodent model of IBS [35,36]. Rats were handled by the same researcher for at least five days prior to behavioral assessment.

In the intervention study, all animal groups (SD: saline, WKY: saline, WKY: Ex-4 and WKY: Ex-4 & xIL-6R; n=12 per group) received an intraperitoneal (IP) injection of anti IgG (Jackson Immunoresearch, Westgrove PA, USA, 1mg/kg,) on day 0 to induce antibody tolerance. For the *in vivo* open field and colorectal distension (CRD) studies, WKY rats were randomly assigned to one of three groups (saline, Ex-4 or Ex- & xIL-6R). SD and WKY rats treated with an IP injection of saline on days 0, 3 and 10 were compared to verify the WKY phenotype of IBS-like bowel dysfunction. The saline-

treated WKY rats were subsequently used as a control for Ex-4 and Ex-4 & xIL-6R treated WKY groups. The GLP-1R agonist, Ex-4 (1.25μg kg^{-1}, Abcam, Cambridge, UK) was administered via IP injection 1 hour prior to the open field trial on day 7 and CRD on day 14 in both groups. In the Ex-4 & xIL-6R group, IP injections of xIL-6R (1mg kg^{-1}, BioRad, Kidlington, OX5 1GE, UK) were delivered on day 0, 3, 6 and 10, as this has previously been shown to be effective in alleviating bowel dysfunction [31]. Weights were monitored at day 0, 3, 6 and 10 during the trial. Whilst a species difference was noted between SD and WKY rats, no difference in weight gain between WKY groups was observed.

Following CRD, rats were euthanized and trunk blood samples were collected in EDTA vacutainer tubes. Samples were centrifuged at 4000 rpm to separate the blood components. Plasma samples were stored at -80°C for later analysis. The distal colon (<4cms from the anus) was excised. For Western blot analysis, mucosal tissue was snap frozen in liquid nitrogen and stored at -80°C.

Open Field Trial

As previously described [35,37], animals were exposed to the psychological stress of being placed in the center of a brightly illuminated (~800 lux), white open field arena, (0.9m in diameter, 38cm in height) for a ten minute trial. Trials were conducted daily between 09.00 and 13.00 and the arena was cleaned with 70% ethanol between trials. The number of fecal pellets excreted during the 10-minute exposure was recorded as an indication of stress-induced defecation. Trials were video-recorded and the distance and velocity of movement was analyzed offline using EthoVision software (Tracksys Ltd, Nottingham, England). The percentage water content of the fecal pellets excreted during

the open field trial was calculated by comparing the wet weight to the dry weigh (dried at 37^{o}C for 24 hours).

Colorectal Distension (CRD)

Rats were fasted for 24 hours before CRD and acclimatized to the testing room for 30 minutes prior to being lightly anaesthetized (inhaled 4% Isoflurane, Abbott Animal research, Ireland). As previously described [37], a latex balloon (6 cm in length, Durex, NJ, USA) was inserted into the colon, 1 cm from the anus. The animals were permitted a recovery time of 10 min before the CRD procedure was initiated. A ramp distension protocol was used where the balloon was distended from 0 to 80mmHg over an 8-minute time period (increasing by 10mmHg each minute) using a customized pressure control device (AstraZeneca R&D, Cambridge, UK). Pain behaviors in conscious rats in response to CRD are identified as contractions of the abdominal and hind limb musculature (abdominal withdrawal reflex), stretching or abdominal retractions [38]. The threshold (mmHg) at which the first visibly identifiable pain behavior and the cumulative number of pain behaviors over the course of the trial was recorded. Offline analysis of recorded behaviors was carried out by a single researcher blinded to the rat strain and intervention.

Western Blotting

As previously described [39], the homogenates of colonic mucosal samples from SD and WKY rats one hour after CRD were denatured and separated on 12% sodium dodecyl sulfate polyacrylamide gels. Proteins were electro-transferred to PVDF membranes (ThermoFisher, Massachusetts, US), which were blocked and incubated with primary antibodies against occludin (Abcam, Cambridge, UK, 1:1,000), claudin-2 (Santa Cruz

Biotechnology, Tx, USA, 1:1,000) and Cav3.2 (Merck Millipore, Temaculla, CA, USA, 1:200, overnight at 4°C). The membranes were subsequently incubated with complimentary horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Westgrove, PA, USA, 1:5,000) before being visualized with an enhanced chemiluminescence detection system (Pierce ECL Western Blotting Substrate, ThermoFisher). Membranes were re-probed with antibodies against β-actin (Cell Signaling Technology, 1:1,000, overnight at 4°C), which acted as a reference protein. Images were captured using a GelDoc Image Reader (Las3000; Fujifilm, Tokyo, Japan) and analyzed using Multigauge v2.2 software (Fujifilm, Tokyo, Japan) to calculate protein densitometry. Protein bands are comparable only between each sub-group, which was conducted at the same time, under identical conditions. Protein samples (n=5) for each experimental group (SD saline, WKY saline, WKY Ex-4, WKY Ex-4 & xIL-6R) were run in a single tank and probed under identical conditions.

Mesoscale Discovery Biomarker Assay

A sandwich immunoassay (V-PLEX Proinflammatory Panel 1 MesoScaleDiagnostics, Gaithersburg, MD, USA) was carried out according to the manufacturer's guidelines to determine concentrations of IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13 and TNFα levels in plasma samples from a subset of treated WKY rats. The test was carried out in duplicate and protein levels were measured using electrochemiluminescence. Plates were read using MesoScale Discovery plate-reader (MESO QuickPlex SQ 120). A standard curve was generated from known cytokine concentrations and sample cytokine concentrations were extrapolated from the curve.

Immunofluorescence & Confocal microscopy

In a separate study in SD rat distal colon, whole-mount preparations of longitudinal muscle myenteric plexus (LMMP) and submucosal plexus (SMP) tissue were prepared from (n=3 different tissue preparations per experiment). Cross sections of the whole colon were cryoprotected in 30% sucrose prior to cryosectioning (10µm in thickness, Leica Biosystems, Wetzler, Germany) and mounted on glass slides (VWR, Dublin 15, Ireland). All tissue was fixed in 4% paraformaldehyde (4^{o}C, overnight), permeabilized with 0.1% Triton X-100 and blocked with 1% donkey serum (all from Sigma Aldrich, UK). The tissues were incubated with primary antibodies (at 4^{o}C overnight) against glucagon-like peptide-1 (GLP-1, 1:250), GLP-1 receptor (GLP-1R, 1:250), interleukin-6 (IL-6, 1:100) or IL-6 receptors (IL-6Rs, 1:250, all from Santa Cruz Biotechnology, Dallas, TX, US) and appropriate FITC- or TRITC- conjugated secondary antibodies (1:250, 2 hours at room temperature, Jackson Immunoresearch, Westgrove, PA, US). Images were captured using a FVl0i-Olympus-confocal microscope with Fluoview software (FV10i-SW). No non-specific fluorescence was detected in control experiments where tissues were incubated with primary antibodies or secondary antibodies alone or where anti GLP-1R and anti-IL-6R antibodies were neutralized with GLP-1 in excess prior to the staining protocol.

Calcium imaging

For calcium imaging studies, a LMMP or SMP tissue preparation from SD distal colon (n=3-4 rats per experiment) was pinned out in Sylgard (Sylgard 184 silicone elastomer kit, WPI, Sarasota FL, USA)-lined petri dishes superfused with carbogen-bubbled Krebs saline solution with 1µM nifedipine to inhibit smooth muscle contractions. The tissue was loaded with Fura-2 AM (7µM, 1 hr, Thermo Fisher Scientific, Waltham, MA, USA) in the dark and washed out prior to recording. Cytosolic changes in intracellular calcium

($[Ca^{2+}]_i$) were recorded from neuronal cell bodies using Cell R software (Olympus Soft imaging solutions, 1986-2009) to record excitation changes in intracellular calcium. Images were acquired at 2Hz using a Xenon/Mercury arc burner (Olympus, Melville, NY, US), a charge-coupled device digital camera (F-view II, Soft imaging system, Munster, Germany) and a 40x water-immersion objective on a fixed stage upright microscope (Olympus BX51WI, South-End-on-Sea, UK). Ganglionic neurons were identified based on morphology and responsivity to brief exposure to 75mM KCl, which was added at the end of each experiment. Responding neurons were defined as those with increases in intracellular calcium $[Ca^{2+}]_i$ greater than two standard deviations from baseline (calculated as the average ratio during the 150 seconds preceding stimulus application). A perfusion system continuously superfused the colonic tissue with carbogen-bubbled Krebs-buffered saline. Ex-4 (10μM, 3min) and IL-6 (1nM, 3 min) were added directly to the superfusate**.**

Statistics

Statistical analysis was carried out using GraphPad prism for Windows (version 5). The data are represented as box and whisker plots showing the median, with 5 and 95% percentile error bars. Student's t-tests and one-way ANOVAs with Tukey's multiple comparison *post-hoc* tests were used where appropriate. Statistical outliers (more than two standard deviations from the mean) were excluded. $P \leq 0.05$ was considered significant.

Results

GLP-1 and IL-6 receptor expression in enteric neurons.

Modulation of intestinal function by the GLP-1 receptor agonist, Ex-4, with or without xIL-6R, is likely to act peripherally, by binding to receptors expressed in gut tissue. Immunofluorescent studies were carried out on colonic tissue from SD rats, which exhibit normal GI function, to ascertain the pattern of expression of GLP-1, IL-6 and their receptors and to assess the potential for crosstalk between the hormone and cytokine. In colonic cross-sections (n=20 sections from 4 rats), epithelial GLP-1-labelled L-cells (green staining, indicated by arrows, figure 1A) were evident. GLP-1 receptor (GLP-1R, red staining) expression was more widespread in the mucosal layer and muscularis and co-localized with GLP-1 in enteric neurons (indicated by arrow, figure 1A) and in a subset of L-cells (n=6 out of 19 L-cells,). Expression of IL-6 (green staining) was strongest in the neuronal plexi (indicated by arrow, figure 1B, n=20 sections from 4 rats) of SD cross-sections. IL-6R expression (red staining) was evident in the muscular layers, epithelial cells and particularly in enteric neurons.

Dual-labelling of myenteric neurons, which regulate GI contractile activity and colonic transit, was carried out to assess the ganglionic expression of GLP-1, IL-6 and their receptors. Distinct subsets of myenteric neurons expressed GLP-1 (red staining) or IL-6 (green staining), although 25% (19/75 neurons in 12 ganglia) of IL-6 positive neurons also expressed GLP-1 (indicated by arrows, figure1C, n=4 LMMP tissue preparations). A different pattern of expression was noted in myenteric ganglia for IL-6 receptors and GLP-1 receptors. IL-6R immunofluorescence (green staining) was strongly localized to neuronal cell bodies. Although GLP-1R immunofluorescence was also present on neuronal cell bodies, it was much more strongly expressed in intra-ganglionic cells

surrounding the neurons (figure 1D, n=4 LMMP tissue preparations). Sensitivity of these neurons to the receptor agonists was confirmed using calcium imaging, where a rise in intracellular Ca^{2+} was interpreted as an indicator of neuronal excitability. Application of the GLP-1R agonist, exendin-4 (Ex-4, 10μM, 3 min) stimulated a robust increase in intracellular calcium ($[Ca^{2+}]_i$) in myenteric neurons (figure 1E). IL-6 (1nM, 3 min) similarly stimulated myenteric neurons although the amplitude of the response was lower as compared to Ex-4 (repeated measures ANOVA, $F(2,27)=2.7$, $p<0.001$). Interestingly, when both IL-6 and Ex-4 were applied together the amplitude of the calcium response was significantly smaller than when the GLP-1 mimetic, Ex-4 was applied alone ($p<0.001$, figure 1E).

Submucosal neurons regulate GI absorption and secretion. Similar to myenteric neurons, submucosal ganglia were immunopositive for GLP-1 and IL-6 protein (n=4 SMP preparations from SD rat colons). A subset (30%, n=26 out of 87 neurons in 12 submucosal ganglia) of IL-6-(green staining) expressing neurons were co-stained with GLP-1 (red staining, indicated by arrow, figure 1F) but many neurons expressed either GLP-1 or IL-6. IL-6R immunofluorescence (green staining) was primarily localized to submucosal neuron cell bodies, whereas GLP-1R expression (red staining) was more strongly expressed in cells surrounding these neurons (n=4 SMP tissue preparations, figure 1G). Submucosal neurons were sensitive to Ex-4 (10μM, 3 min), responding with an increase in $[Ca^{2+}]_i$. IL-6 (1nM, 3 min) evoked a calcium response in the same neurons, although this was smaller in amplitude than the Ex-4-evoked response (repeated measures ANOVA, $F(2,53)=64.7$, $p<0.001$). However, when both were applied together the response was larger than either Ex-4 ($p<0.001$) or IL-6 ($p<0.001$, figure 1H), alone.

A GLP-1 mimetic normalizes stress-induced defecation in WKY rats.

IBS-like traits exhibited by stress-sensitive WKY rats include augmented colonic transit, expressed as increased defecation rates, in response to an environmental stressor [35]. Our cohort of saline-treated WKY rats (n=12) displayed increased stress-induced defecation in the open field arena as compared to saline-treated SD rats (n=11, $p<0.0001$, Student's t-test, figure 2). To investigate the potential of GLP-1R agonists to inhibit bowel motility [14], WKY rats were treated with Ex-4 (1.25µg/kg I.P., 1 hour prior to the open field trial), in the absence or presence of xIL-6R (1mg/kg, four I.P. injections over two weeks) and assessed in the open field arena. When compared to saline-treated WKY rats (n=10), Ex-4 treatment reduced the number of fecal boli excreted in the anxiogenic environment of the open field arena (n=12, $p<0.0001$, one-way ANOVA with Tukey post-test). However, no additive effect was noted in WKY rats co-treated with xIL-6R (n=11, figure 2).

Ex-4 treated WKY rats exhibited modified mucosal expression of tight junction proteins.

To determine whether stimulation of submucosal neurons by Ex-4 resulted in modulation of GI absorpto-secretory function, we examined the water content of the fecal pellets excreted in the open field arena. No significant difference was detected between saline-treated SD (n=5) and saline-treated WKY rats (n=9, Student's t-test, $p=0.123$). Moreover, *in vivo* treatment with Ex-4 in the absence (n=6) or presence of xIL-6R (n=4), had no impact on water content in WKY fecal pellets ($p=0.253$, one-way ANOVA with Tukey post-test, figure 3A).

As tight junction proteins are also important in intestinal barrier permeability, protein expression of claudin-2 and occludin from homogenized colonic mucosal tissue was compared between experimental groups. Increased expression of claudin-2 is thought to increase barrier permeability [40], but, as compared to SD rats (n=5) expression of this protein was reduced in WKY colons (n=5, p=0.043, Student's t-test). No change in expression of claudin-2 was evident in WKY colonic mucosa from rats administered Ex-4 (n=5) or Ex-4 with xIL-6R (n=5 rats, p=0.137, one-way ANOVA with Tukey post-test, figure 3B). Depletion of the tight junction protein, occludin is linked with increased intestinal barrier permeability [41], however, no change in mucosal expression of occludin was noted between SD (n=4) and WKY rats (n=5, p=0.15, Student's t-test,). Nonetheless, WKY rats treated with Ex-4 (n=4), but not Ex-4 and xIL-6R (n=5), had lower expression of occludin (p=0.011, figure 3C).

Visceral hypersensitivity in WKY rats is not evident following Ex-4 treatment.

Consistent with previous reports of visceral hypersensitivity to CRD (Gibney et al., 2010; Gunter et al., 2000; O'Mahony et al., 2010), we observed that the threshold at which CRD (0-80mmHg over 8 minutes) evoked pain behaviors was lower in WKY rats (n=10) than SD rats (n=9, p=0.02, Student's t-test, figure 4A). There was no significant difference in the pain threshold to CRD between saline-treated (n=10), Ex-4-treated (n=11) or Ex-4 plus xIL-6R-treated (n=10) WKY rats (p=0.439, one-way ANOVA with Tukey post-test, figure 4A). However, in WKY rats treated with Ex-4 in the presence or absence of xIL-6R, the threshold to the first pain behavior were not different to the SD control rats (p=0.556, one-way ANOVA with Tukey post-test). Consistent with the link between expression of $Ca_v3.2$, T-type calcium channels and visceral pain sensitivity [42], we found that mucosal expression of $Ca_v3.2$ was increased in saline-treated WKY rats

(n=4) as compared to SD controls (n=5, p=0.05, Student's t-test, figure 4B). However, mucosal expression $Ca_v3.2$ was not different in WKY rats treated with Ex-4 (n=4) or co-treatment with Ex-4 plus xIL-6R (n=5) as compared to saline-treated WKY rats (p=0.151, one-way ANOVA with Tukey post-test).

Intraperitoneal Ex-4 does not modify anxiety-like behaviors in WKY rats.

The sensitivity of WKY rats to stress was evident by the display of anxiety-like behaviors in the open field arena. WKY rats (n=11) were less inclined to explore the exposed inner zone of the open field arena compared to SD controls (n=11), spending less time in the inner zone (p=0.015, Student's t-test, figure 5A) and making fewer entries into the exposed inner zone (p=0.0002, Student's t-test, figure 5B). Time spent in the inner circle of the open field arena by WKY rats treated with Ex-4 (n=11) or Ex-4 plus xIL-6R (n=11) remained significantly lower than control SD rats (p=0.0114, one-way ANOVA with Tukey post-test, figure 5A). Similarly, the number of entries to the central region by treated WKY rats was significantly less than SD controls (p<0.0001, one-way ANOVA with Tukey post-test, figure 5B).

Ex-4 increased circulating levels of anti-inflammatory IL-13 in WKY rats.

Shortening of colon length can indicate inflammation [43] and saline-treated WKY rats (n=11) had shorter colons than SD comparators (n=11, p= 0.009, Student's t-test, figure 6). Colonic length in WKY rats treated with Ex-4 (n=10) or co-treated with Ex-4 plus xIL-6R (n=11) did not show any significant difference to saline-treated WKY rats (p=0.858, one-way ANOVA with Tukey post-test, figure 6) and tended towards being shorter than SD controls (p=0.087, one-way ANOVA with Tukey post-test).

Circulating pro- and anti-inflammatory cytokines were compared between saline-treated WKY rats and those treated with Ex-4 or Ex-4 plus xIL-6R (n= plasma from 4-6 rats per group). TNFα, a pro-inflammatory cytokine and IL-4, an anti-inflammatory cytokine, were not detectable in any of the plasma samples. No significant changes in circulating levels of pro-inflammatory IFNγ, IL-1β, IL-6 or IL-8 were detected ($p>0.05$, one-way ANOVA with Tukey post-test, table 1). The anti-inflammatory cytokines, IL-2 ($p=0.227$, one-way ANOVA with Tukey post-test), IL-10 ($p=0.161$, one-way ANOVA with Tukey post-test) and IL-12p70 ($p=0.313$, one-way ANOVA with Tukey post-test, table 1) were not modified by the treatments. However, Ex-4 induced an increase in circulating levels of the anti-inflammatory cytokine, IL-13 ($p=0.022$, one-way ANOVA with Tukey post-test, table 1).

Discussion

Ingestion of food has been linked to the genesis of IBS symptoms such as abdominal bloating and diarrhea [7-9]. The onset or deterioration of symptoms can become apparent in up to 60% of IBS patients within 15 – 180 minutes of food intake [44] . Possible mechanisms by which nutrients induce GI symptoms include activation of mechanoreceptors due to gut distension, activation of immune cells, nutrient sensing by enteroendocrine cells or a combination of these [45]. Our study has used a pre-clinical rat model of IBS to investigate mechanisms underlying the reported beneficial effects of administering GLP-1R agonists to patients with functional bowel disorders [17]. We also investigated whether co-treatment with neutralizing IL-6R antibodies, which we have previously shown to improve IBS-like symptoms in WKY rats [32], enhanced the therapeutic efficacy of administering a GLP-1 mimetic.

GLP-1 can act as a classic hormone, a paracrine molecule or a neuromodulatory factor. The peak of circulating GLP-1 levels coincides with post-prandial symptom manifestation in IBS patients [12] and in addition to its role as an incretin hormone, GLP-1 also modifies GI function [13], both in healthy controls [46] and in patients with IBS [14-16]. Given our previous work [10], where we determined that fasting GLP-1 levels are decreased in diarrhea-predominant IBS patients, one could propose that supplementing patients with GLP-1 could be beneficial in improving GI symptoms [17]. In contrast, circulating levels of the pro-inflammatory cytokine, IL-6, which can modify neural regulation of gut function [31,36,47], is elevated in diarrhea-predominant IBS patients [26]. In the WKY rat model, which exhibits IBS-like symptoms, inhibition of IL-6 signaling improved visceral pain sensitivity and stress-induced defecation, although these symptoms were not normalized [31]. Given the multi-factorial nature of IBS

pathophysiology, post-prandial secretion of GLP-1 in conjunction with low-grade immune activation typified by elevated levels of IL-6, could contribute to symptom exacerbation. Thus, the therapeutic potential of a GLP-1 mimetic alone and in conjunction with neutralizing IL-6R antibodies was investigated in the WKY rat model of IBS.

WKY rats exhibited anxious behaviors such as freezing and avoiding the exposed inner zone of the arena of the open field arena and, although the water content was not significantly increased, they defecated more often than their control comparators. Although the half-life of Ex-4 is longer than GLP-1, which is rapidly degraded by dipeptidyl peptidase-4, subcutaneously injected Ex-4 is reported to only have a half-life of ~70 minutes [48]. Therefore, in our studies Ex-4 was administered one hour prior to behavioral assessments. Decreased excretion of fecal boli in Ex-4 treated WKY rats is consistent with reports in humans that Ex-4 mollified colonic transit [14-16,46]. We have previously established the intricate relationship between hormones and immune molecules and demonstrated the efficacy of xIL-6R in reducing stress-induced defecation in WKY rats [31]. However, in this study, no further change in stress-induced defecation was observed in WKY rats co- treated with xIL-6R.

Interestingly, whilst both GLP-1 and IL-6 protein and receptors were detected in healthy SD myenteric neurons, distinct patterns of expression were evident, with minimal co-localization of neurons expressing protein or receptors. This may indicate that different population of neurons, which may be stimulatory or inhibitory may be activated by the receptor agonists. IL-6Rs were localized to myenteric neuron cell bodies, whereas punctate GLP-1R expression was stronger in intra-ganglionic cells surrounding the neurons. Glial cells surround ganglionic neurons and are known to

actively modulate neuronal excitability, however, GLP-1R expression was not detected in S100-labelled myenteric glial cells [49]. The immunofluorescently-visualized GLP-1 receptors may therefore be predominantly expressed on innervating fibers. Indeed, GLP-1Rs have been detected in human colonic nerve fibers and dorsal root ganglion cells [50]. Interestingly, the number of GLP-1R-positive fibers is increased in patients with inflammatory bowel disease [50], which, similar to IBS, is characterized by elevated levels of pro-inflammatory cytokines such as IL-6 [51]. It is interesting, given that GLP-1 and IL-6 receptors are not commonly expressed in the same cells, that cross-talk is apparent in the neurostimulatory actions of the receptor agonists [31,49]. The amplitude of the calcium response evoked by Ex-4 is suppressed when IL-6 is co-applied. Although this relationship needs to be researched further, neuronal cross-talk between these molecules could impact of colonic function. In submucosal neurons which regulate intestinal absorption and secretion, protein expression of IL-6 and GLP-1 was also commonly detected in separate groups of neurons. IL-6R expression was typically found in neuronal cell bodies, whereas GLP-1R expression was strongest in the intraganglionic cells surrounding the neurons, which could include glial cells or innervating fibers. Similar to previous reports in acute preparations of submucosal neurons, application of both Ex-4 [10] and IL-6 [22] evoked calcium responses. However, in contrast to the effects in myenteric neurons, when Ex-4 was applied in conjunction with IL-6, the amplitude of the calcium response was larger than the control responses, indicating the relationship between these molecules differs between neuronal plexi, which could further impact on colonic function.

Epithelial expression of tight junction proteins which regulate barrier permeability, was altered in WKY rats as compared to SD controls. Increased expression of claudin-2 is

thought to increase barrier leakiness [40], whereas we noted decreased expression in saline-treated WKY rats . Moreover, there is a trend towards increased expression of occludin in WKY rats, when depletion of this tight junction protein is linked with increased intestinal barrier permeability [41]. This suggests that the colonic epithelial barrier in WKY rats is less permeable that the SD control which is consistent with evidence that demonstrated that baseline colonic ion transport and cholinergic-induced ion transport was decreased in WKY rats as compared to SD rats [52]. Claudin-2 expression was not modified in either treatment group but interestingly, colonic mucosa from WKY rats treated with Ex-4 alone, but not Ex-4 and xIL-6R had reduced expression of occludin, suggesting that Ex-4 could modify barrier permeability. Nonetheless, the GLP-1 mimetic had no significant effect on water content of fecal pellets excreted by WKY rats.

Pain threshold to CRD in saline-treated WKY rats was not significantly different to WKY rats treated with Ex-4 alone, or with Ex-4 and xIL-6R. However, these two groups were not significantly different to SD controls, suggesting some improvement in these groups. $Ca_v3.2$ T-type calcium channels are linked to visceral pain sensitivity [42] and similar to previous studies [31], mucosal expression of these channels were elevated in WKY rats. However, expression of this channel was not modified by either treatment. Although GLP-1Rs are present in the CNS, and GLP-1 can cross the blood brain barrier [53], no changes in centrally-regulated anxiety-like behaviors in WKY rats were apparent following treatments.

Consistent with the multi-factorial nature of IBS, interplay between endocrine and immune factors contribute to bowel dysfunction in WKY rats [31]. Several studies have

reported altered cytokine profiles in IBS [24,25,54] and WKY rats [55], where stress hormones and immune cytokines were investigated in relation to the pathophysiology of depression. Ex-4 has anti-inflammatory effects in the intestine [56] and may suppress pro-inflammatory cytokines in human obese and diabetic patients [57]. Shortening of colon length can be indicative of inflammation [43]. Indeed, we previously reported altered colon lengths in two models of bowel dysfunction [35]. In this cohort of WKY rats, colonic length was shorter than SD rats, suggesting immune activation. However, this was not significantly altered by treatment with Ex-4 or Ex-4 plus xIL-6R. The *in vivo* treatments did not alter any of the pro-inflammatory cytokines in WKY rats however, circulating levels of the anti-inflammatory Th2 cytokine, IL-13 was elevated following Ex-4 treatment. IL-13 has been reported to induce the replacement of epithelial cells with goblet cells, an observation that we have previously reported in the proximal colon of WKY rats [35] and elevated levels of this cytokine are consistent with the purported anti-inflammatory actions of GLP-1 [58].

Conclusions.

Administration of a GLP-1 mimetic, Ex-4, normalized stress-induced defecation in the WKY rat model of bowel dysfunction, with more modest effects on visceral pain sensitivity and immune activation and no changes in centrally-orchestrated anxiety-like behaviors. Modulation of enteric neuronal function and tight junction expression appear to underlie the functional benefits of this intervention. Although we have previously reported that gut dysfunction in this animal model can be improved by *in vivo* treatment with xIL-6R, no additive effect was noted when WKY rats were co-treated with Ex-4 and xIL-6R. These studies begin to elucidate the mechanisms underlying the beneficial effects of GLP-1 mimetics in functional bowel disorders.

Acknowledgements

The authors wish to thank Dr M. Buckley, Ms. Louise O'Sullivan and Ms. Amy Kelliher for their help with this study.

The authors declare that there are no competing interests regarding this publication.

Author Contribution:

RO'B: Generation and analysis of data. Drafting of manuscript.

DO'M: Designed the research study, reviewed the manuscript, sourced funding.

Grant support: This work was supported by a grant from TRAP funding, School of Medicine, University College Cork, Ireland. RO'B is supported by the Department of Physiology, University College Cork, Ireland.

Abbreviations:

Ca^{2+}, calcium; CRD, colorectal distension; Ex-4, Exendin-4; GI, gastrointestinal; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; IBS, irritable bowel syndrome; IL, interleukin; IL-6R, interleukin-6 receptor; intracellular Ca^{2+}, $[Ca^{2+}]_i$; IP, intraperitoneal; LMMP, longitudinal muscle myenteric plexus; SD, Sprague Dawley; SMP, submucosal plexus; WKY, Wistar Kyoto; xIL-6R, anti-IL-6R monoclonal antibody.

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Table 1: Treatment-evoked changes in immune cytokines in WKY plasma.

Pro-inflammatory cytokines in WKY plasma				
	Saline treated (pg ml^{-1})	**Ex-4 treated (pg ml^{-1})**	**Ex-4 & xIL-6R treated (pg ml^{-1})**	**P value (One-way ANOVA)**
IFN-γ	0.51 ±0.03	0.58 ±0.04	0.63 ±0.05	P=0.18
IL-1β	0.87 ±0.06	0.86 ±0.09	0.64 ±0.11	P=0.18
IL-6	0.01 ±0.01	0.016 ±0.007	0.022 ±0.009	P=0.15
IL-8	0.27 ±0.075	0.21 ±0.03	0.19 ±0.01	P=0.34
TNFα	ND	ND	ND	
Anti-inflammatory cytokines in WKY plasma				
	Saline treated (pg ml^{-1})	**Ex-4 treated (pg ml^{-1})**	**Ex-4 & xIL-6R treated (pg ml^{-1})**	**P values (One-way ANOVA)**
IL-2	0.18 ±0.006	0.16 ±0.01	0.15 ±0.004	P=0.22
IL-4	ND	ND	ND	
IL-10	0.03 ±0.006	0.02 ±0.002	0.04 ±0.01	P=0.16
IL-12p70	0.082 ±0.008	0.094 ±0.005	0.09 ±0.004	P=0.31
IL-13	0.15 ±0.007	0.18 ±0.005*	0.16 ±0.006	P=0.02

Figure and Table Legends

Figure 1: Expression of IL-6 and GLP-1 in the distal colon

A: The representative immunofluorescent images in cross sections from the distal colon of Sprague Dawley (SD) rats illustrate expression of glucagon-like peptide-1 (GLP-1, green staining) in mucosal L-cells (indicated by arrows) and GLP-1 receptors (GLP-1R, red staining). **B:** Interleukin-6 (IL-6, green staining) and IL-6 receptor (IL-6R, red staining) immunofluorescence were highly expressed in enteric ganglia (indicated by arrows). Scale bars: 200μm. **C:** The representative immunofluorescent images show protein expression of IL-6 (green staining) and GLP-1 (red staining) in myenteric neurons. Some neurons expressed both proteins (indicated by arrows). **D:** Distinct IL-6R (green staining) and GLP-1R (red staining) expression were evident in SD distal colonic myenteric ganglia. Scale bars: 20μm. **E:** The box and whisker plot and representative calcium trace illustrates the neurostimulatory actions of Ex-4 and IL-6 in myenteric neurons. **F:** The representative immunofluorescent images show protein expression of IL-6 (green staining) and GLP-1 (red staining) and **G:** IL-6R (green staining) and GLP-1R (red staining) in SD distal colonic submucosal ganglia. Scale bars: 20μm. **H:** The box and whisker plot and representative calcium trace illustrates the neurostimulatory actions of exendin-4 (Ex-4) and IL-6 in SD submucosal neurons. *** indicates $p<0.001$.

Figure 2: Ex-4 inhibits stress-induced defecation in the Wister Kyoto rat model of IBS.

The box and whisker plots illustrate the number of fecal boli excreted in the open field arena by Sprague Dawley (SD) and Wister Kyoto (WKY) rats treated with saline,

exendin-4 (Ex-4) or Ex-4 & anti-interleukin-6 receptor monoclonal antibodies (xIL-6R). *** indicates $p<0.001$.

Figure 3: Mucosal expression of tight junction proteins was modified by Ex-4 treatment.

A: The water content in fecal boli excreted in the open field arena by Sprague Dawley (SD) and Wister Kyoto (WKY) rats treated with saline, Ex-4 or Ex-4 & anti-interleukin-6 receptor monoclonal antibodies (xIL-6R) was comparable. **B:** The box and whisker plots and the representative Western blots illustrate relative protein expression (calculated as a ratio of β-actin expression) of the tight junction proteins, Claudin-2 and **C:** occludin in each experimental group. * indicates $p<0.05$.

Figure 4: Visceral hypersensitivity in WKY rats is not evident following treatment with Ex-4.

A: The box and whisker plots illustrate the pressure ($mmHg^{-1}$) at which Sprague Dawley (SD) and Wister Kyoto (WKY) rats treated with saline, Exendin-4 (Ex-4) or Ex-4 & anti-interleukin-6 receptor monoclonal antibodies (xIL-6R) exhibit their first pain behavior in response to colorectal distention. **B:** The plots and representative blots show mucosal expression of the T-type calcium channels, $Ca_v3.2$, in SD rats and WKY rats treated with Ex-4 alone or Ex-4 combined with xIL-6R. * indicates $p<0.05$. NS indicates non-significant.

Figure 5: The anxious phenotype of Wister Kyoto rats in unaffected by Ex-4 or xIL-6R.

A: The box and whisker plot illustrates anxiety-like behaviors in Wister Kyoto (WKY) rats as compared to Sprague Dawley (SD) controls. WKY rats spent less time in the anxiogenic inner circle of the open field arena and **B:** made fewer exploratory entries to the inner zone. This was not modified by treatment with exendin-4 (Ex-4) or co-treatment with Ex-4 and anti-interleukin-6 receptor monoclonal antibodies (xIL-6R). * ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Figure 6: Shortened colon length in WKY rats is normalized by Ex-4 treatment.
The box and whisker plot shows colon length is decreased in saline-treated Wister Kyoto (WKY) rats as compared to Sprague Dawley (SD) controls. WKY colons from rats treated with exendin-4 (Ex-4) and Ex-4 plus anti-interleukin-6 receptor monoclonal antibodies (xIL-6R) were not significantly different to saline-treated WKY controls. ** indicates $p<0.01$.

Table 1

The table illustrates circulating levels of pro- and anti-inflammatory cytokines in plasma samples taken from Wister Kyoto (WKY) rats administered saline, exendin-4 (Ex-4) or Ex-4 plus neutralizing anti-IL-6 receptor antibodies (xIL-6R) *in vivo*. ND: not detected.

Figure 1

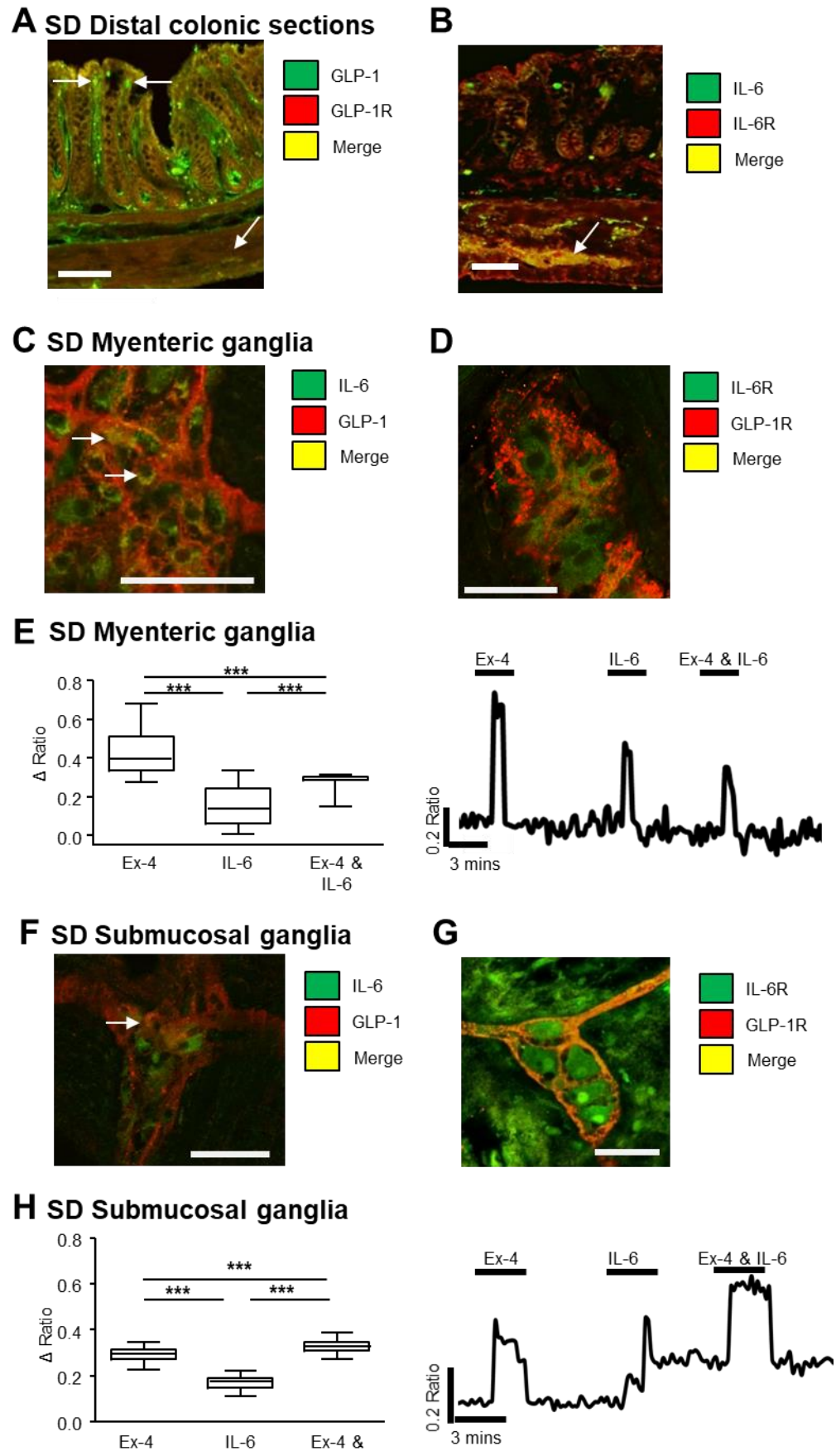

A SD Distal colonic sections
GLP-1
GLP-1R
Merge
B
IL-6
IL-6R
Merge
C SD Myenteric ganglia
IL-6
GLP-1
Merge
D
IL-6R
GLP-1R
Merge
E SD Myenteric ganglia
***
***
***
Δ Ratio
0.8
0.6
0.4
0.2
0.0
Ex-4
IL-6
Ex-4 & IL-6
Ex-4
IL-6
Ex-4 & IL-6
0.2 Ratio
3 mins
F SD Submucosal ganglia
IL-6
GLP-1
Merge
G
IL-6R
GLP-1R
Merge
H SD Submucosal ganglia
***
***
***
Δ Ratio
0.8
0.6
0.4
0.2
0.0
Ex-4
IL-6
Ex-4 & IL-6
Ex-4
IL-6
Ex-4 & IL-6
0.2 Ratio
3 mins

Figure 2

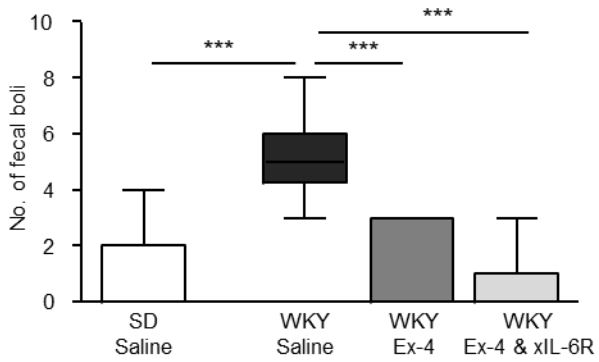


Figure 3

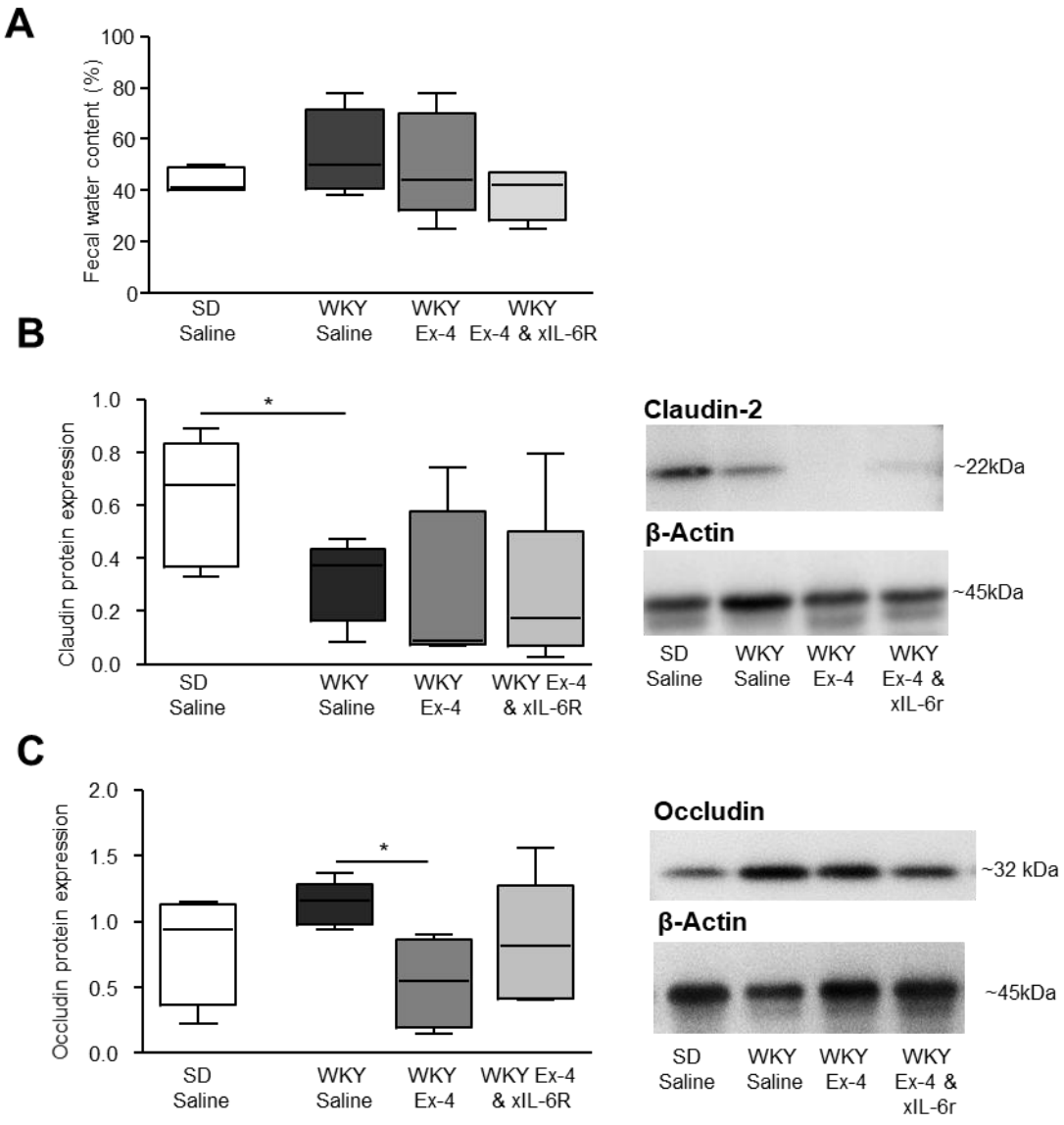


Figure 4

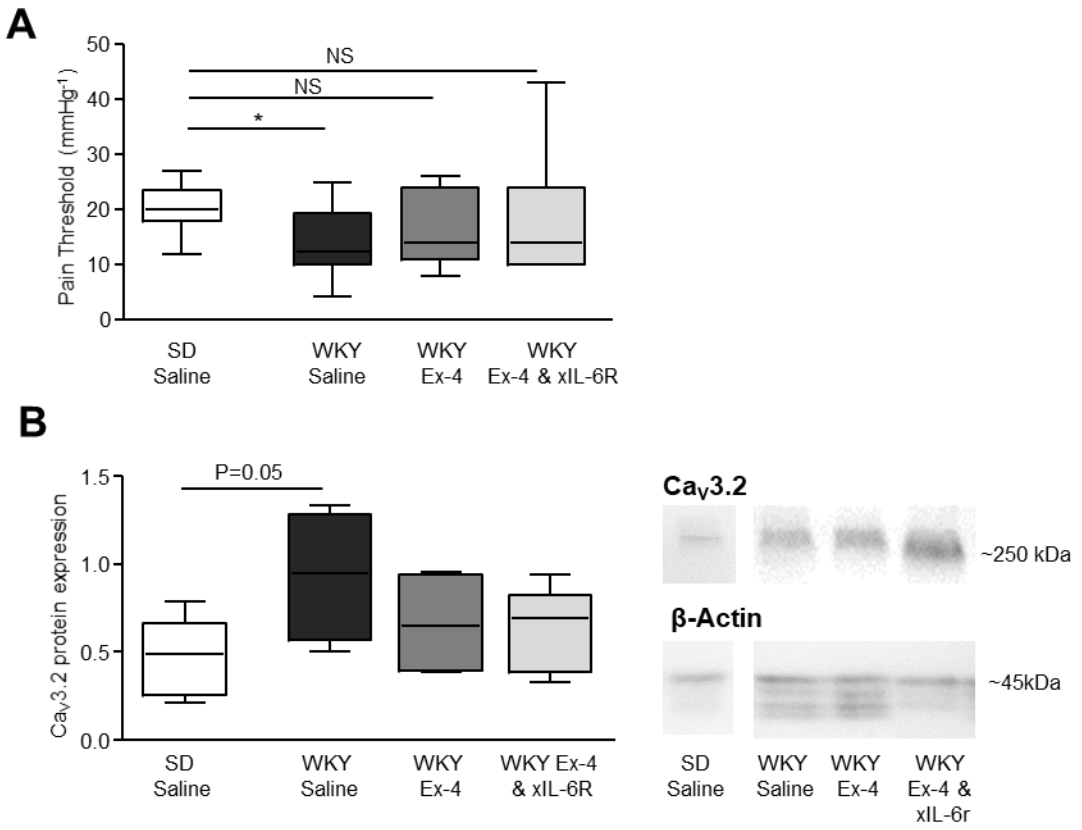


Figure 5

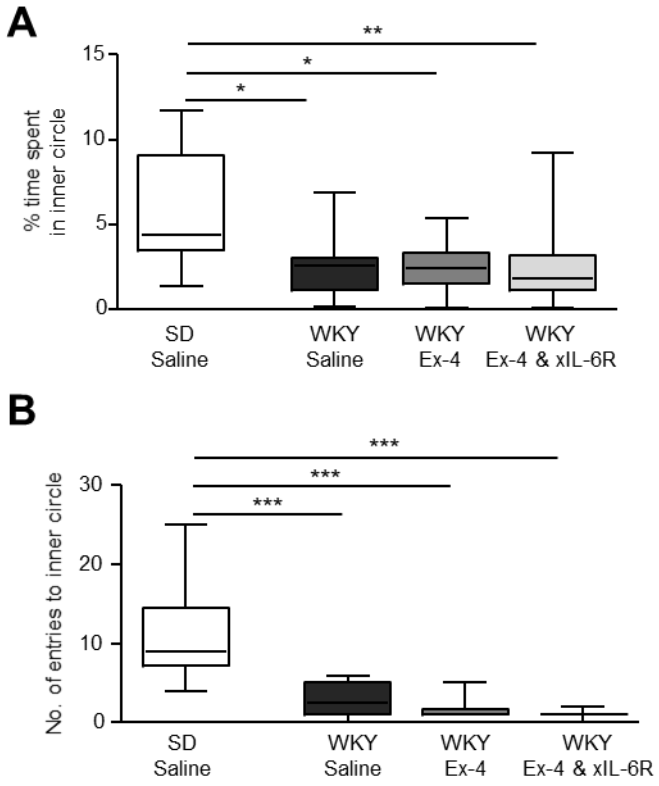
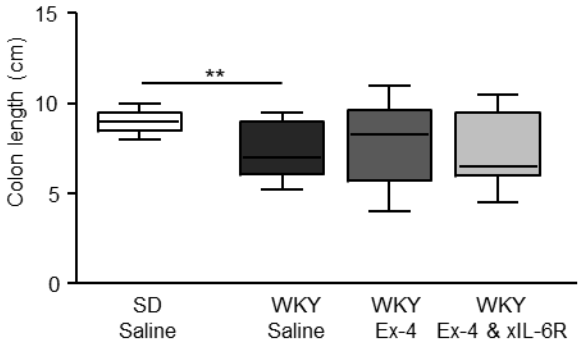


Figure 6


15
10
5
0
Colon length (cm)
**
SD
Saline
WKY
Saline
WKY
Ex-4
WKY
Ex-4 & xIL-6R